

Effect of advanced glycation end-products (AGE) lowering drug ALT-711 on biochemical, vascular, and bone parameters in a rat model of CKD-MBD

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Abstract: Chronic Kidney Disease-Mineral Bone Disorder (CKD-MBD) is a systemic disorder that affects blood measures of bone and mineral homeostasis, vascular calcification and bone. We hypothesized that the accumulation of advanced glycation end-products (AGE) in CKD may be responsible for the vascular and bone pathologies via alteration of collagen. We treated a naturally occurring model of CKD-MBD, the Cy/+ rat, with a normal and high dose of the AGE crosslink breaker alagebrium (ALT-711), or with calcium in the drinking water to mimic calcium phosphate binders for 10 weeks. These animals were compared to Normal (NL) untreated animals. The results showed that CKD animals, compared to normal animals, had elevated BUN, PTH, FGF23 and phosphorus. Treatment with ALT-711 had no effect on kidney function or PTH, but 3 mg/kg lowered FGF23 whereas calcium lowered PTH. Vascular calcification of the aorta assessed biochemically was increased in CKD animals compared to NL, and decreased by the normal, but not high dose of ALT-711, with parallel decreases in left ventricular hypertrophy. ALT-711 (3 mg/kg) did not alter aorta AGE content, but reduced aorta expression of receptor for advanced glycation end products (RAGE) and NADPH oxidase 2 (NOX2), suggesting effects related to decreased oxidative stress at the cellular level. The elevated total bone AGE was decreased by 3 mg/kg ALT-711 and both bone AGE and cortical porosity were decreased by calcium treatment, but only calcium improved bone properties. In summary, treatment of CKD-MBD with an AGE breaker ALT-711, decreased FGF23, reduced aorta calcification, and reduced total bone AGE without improvement of bone mechanics. These results suggest little effect of ALT-711 on collagen, but potential cellular effects. The data also highlights the need to better measure specific types of AGE proteins at the tissue level in order to fully elucidate the impact of AGEs on CKD-MBD.

Key Words: Bone QCT/ μ CT, chronic kidney disease-mineral bone disorder (CKD-MBD),
vascular calcification, bone quality

Introduction:

Chronic Kidney Disease (CKD)- Mineral Bone Disorder (CKD-MBD) is a systemic disorder manifested by abnormal mineral metabolism, vascular calcification, and abnormal bone(1). The consequences of CKD-MBD include increased cardiovascular events from left ventricular hypertrophy and arterial calcification(2), elevated parathyroid hormone (PTH) and fibroblast growth factor-23 (FGF23)(3, 4), and increased risk of fracture(5) due to abnormal bone remodeling and increased cortical porosity. All three end organ manifestations of CKD-MBD (arteries, heart, and bone) undergo collagen turnover/remodeling as a normal physiologic response. However, pathologic alterations in collagen may affect this remodeling and lead to aberrant architecture and abnormal function.

In diabetes, aging, and CKD, collagen may be altered with advanced glycation end-products (AGEs). AGEs represent a heterogeneous group of macromolecules that are formed by the non-enzymatic glycation (Amadori rearrangement of a Schiff base) of proteins, lipids, and nucleic acids. AGEs accumulate due to elevated blood glucose, from ingestion of food altered with AGEs, and as both downstream and upstream consequences of increased oxidative stress(6). The most commonly measured AGEs, pentosidine and N(6)-carboxymethyllysine (CML), are increased in the circulation and skin and associated with cardiovascular mortality in patients with CKD(7, 8). AGEs are associated with arterial calcification in patients with CKD(9) and induce calcification of vascular smooth muscles in vitro(10, 11) and in animal models(12). In bone, studies in older patients with post-menopausal osteoporosis have shown increased AGE accumulation and association with bone fragility(13). In humans(14) and in animals with CKD(15) AGEs lead to abnormal mineralization. These data suggest that AGEs play a

pathogenic role in multiple components of CKD-MBD and thus a reduction in AGE collagen cross links may improve the systemic manifestations of CKD-MBD.

We therefore tested that hypothesis that ALT-711 (alagebrium), a compound that has been shown to effectively lower AGEs in kidney(16), aorta(12), and heart(17) of animals with diabetic nephropathy(18), would ameliorate consequences of CKD-MBD. To test this hypothesis, we used our slowly progressive, naturally occurring, Cy/+ rat model that spontaneously develops arterial calcification, left ventricular hypertrophy, and compromised bone phenotypes (increased cortical porosity and reduced mechanical properties).

Material and Methods:

Experimental design

Cy/+ rats are characterized by an autosomal dominant progressive cystic kidney disease. The animals have a mutation in *Anks6*, a gene that codes for the protein SamCystin located at the base of cilia. Although specific function is not fully elucidated, *Anks6* binds with *Anks3* and *Bicc1* and thus may alter the nephronophthisis complex(19, 20), but the cilia are not directly affected. In this rat model, CKD-MBD develops spontaneously, with a much faster progression to end stage disease in male animals by 30 to 40 weeks of age, whereas female rats do not develop azotemia even as old as 21 months(21), or after oophorectomy(22). The Cy/+^{IU} colony of rats has been bred at Indiana University for nearly 20 years. For the present study, male Cy/+^{IU} rats (n=56 hereafter called CKD) and unaffected Sprague Dawley normal littermates (NL; n=14) were placed on a casein diet (Purina AIN-76A; 0.7% Pi, 0.6% Ca) at 24 weeks of age in order to produce a more consistent CKD-MBD phenotype [38]. Treatment began at 25 weeks of age (~ 50% normal GFR), and at 35 weeks of age (~15% normal GFR), animals were

anesthetized with isoflurane and underwent cardiac puncture for blood collection followed by exsanguination and bilateral pneumothorax to ensure death. All animals received an injection of calcein (30 mg/kg) fourteen and four days prior to euthanasia. Heart, aorta, kidney, tibia and femora were collected, weighed as appropriate, and stored for analysis. Left ventricular mass index (LVMI) was determined by dividing total heart weight by body weight.

Multiple studies have tested the effect of ALT-711 in rodent models, using doses of 1 to 10 mg/kg daily for up to 10 weeks(12, 23-25) and in human studies assessing vascular function, doses of 2.8 to 6 mg/kg (assuming 70 kg person) have been used for up to one year(26-28). We therefore chose two doses of ALT-711: 3 mg/kg for efficacy and 15 mg/kg for toxicity. Five groups of animals were compared (n = 14 at start of treatment): 1) Normal littermate animals, 2) CKD animals without treatment, 3) CKD animals treated with normal doses of ALT-711 (3mg/kg i.p. daily) for 10 weeks, 4) CKD animals treated with high doses of ALT-711 (15mg/kg i.p. daily) for 10 weeks, and 5) a group of CKD animals given 3% calcium gluconate in the drinking water to simulate a calcium based phosphate binder and lower PTH, inducing a low bone turnover phenotype(29). One of the calcium treated animals, and one of the normal dose ALT-711 treated animals died prior to euthanasia. All procedures were approved by, and carried out according to the rules and regulations of, the Indiana University School of Medicine's Institutional Animal Care and Use Committee.

Blood Biochemistry:

Blood plasma was analyzed for BUN, creatinine, calcium, and phosphorus using colorimetric assays (Point Scientific, Canton, MI, or BioAssay Systems, Hayward, CA). Intact PTH and serum C-terminal FGF23 were determined by ELISA kits (Quidel, San Diego, CA). Serum levels of an oxidative stress marker, 8-hydroxy-2'-deoxyguanosine (8-OHdG) were

measured using an ELISA kit (Enzo Life Sciences, Farmingdale, NY) and TBARS were measured using TBARS assay (TCA method) kit (Cayman Chemical, Ann Arbor, MI). Serum inflammation marker C-reactive protein (CRP) was measured by ELISA (Alpha Diagnostic International, San Antonio TX). Serum AGE levels were determined by OxiSelect Advanced End Glycation product Competitive ELISA (Cell BIOLABS, San Diego, CA). Total serum iron levels and total iron binding capacity (TIBC) were determined by the Endocrinology core lab at the Indiana University School of Medicine.

Effect of ALT on in vitro AGE-collage formation:

In order to confirm that ALT-711 alters collagen AGE formation, Type I collagen was solubilized in sterile 0.01 N acetic acid (0.1 $\mu\text{g}/\mu\text{l}$), poured into culture dishes (100 $\mu\text{g}/\text{cm}^2$), and dried at room temperature overnight. The collagen formed was further incubated with or without 100 mM ribose in the presence or absence of ALT-711 (2 mM) for 3 weeks in a sterile condition. AGE-collagen was qualitatively examined by fluorescence (356 nm excitation /440 nm emission) by confocal microscope (30).

Measurement of AGEs in tissue:

The femoral shaft was flushed with saline to remove marrow followed by incubating with Immunocal (Decal Chemical Corporation, Congers, NY) to demineralize the bone for 2-3 days. Demineralization end-point determination assays (Polysciences, Warrington, PA) were used to verify demineralization of each specimen. The demineralized bone tissues were then hydrolyzed in 6 M HCL at 110°C for 16 hours(31). AGE content was determined using fluorescence readings taken with a CLARIOstar high performance microplate reader (BMG LABTECH Inc, Cary, NC) at wavelengths of 370nm/440nm excitation/emission against a quinine sulfate standard(32)and normalized by the collagen content for the sample. The amount of collagen for

each bone specimen was based on the amount of hydroxyproline determined by a hydroxyproline assay kit according to manufactures' instruction (Sigma-Aldrich USA, St. Louis, MO). AGE levels in heart and kidney were similarly measured.

Bone AGEs were also assessed by HPLC. After mechanical testing, segments of the femoral cortex (~3 mm in length) were processed for assessment of enzymatic collagen crosslinks and AGEs (pentosidine) by a high performance liquid chromatography (HPLC) system (Beckman-Coulter System Gold 168) as previously published(33). Standards of pyridinoline (PYD; Quidel), deoxypyridinoline (DPD; Quidel), pentosidine (PE; International Maillard Reaction Society) were used. The amount of collagen for each bone specimen was based on the amount of hydroxyproline determined by a hydroxyproline assay kit (Sigma-Aldrich USA, St. Louis, MO) and was used to normalize crosslink concentration (mol/mol collagen).

Aortic arch calcification:

To quantify aortic calcification, segments of aortic arches were incubated in 0.6 N HCl for 48 h and the supernatant analyzed for calcium using the *o*-cresolphthalein complex 1 method (Calcium kit; Pointe Scientific) and normalized by tissue dry weight as previously described (34).

RNA isolation, quantification and real-time PCR:

Total RNA from aorta was isolated using miRNeasy Mini Kit (Qiagen). Target-specific PCR primers were obtained from Applied Biosystems. The gene expression of receptor for AGE (RAGE) and NADPH oxidase isoform 2 and 4 (NOX2 and 4) was analyzed by real time PCR using Taqman gene expression assay system (TaqMan MGP probes, FAM dye-labeled , Applied Biosystems, Foster City, CA) using ViiA 7 systems(35). The cycle number at which the

amplification plot crosses the threshold was calculated (C_T), and the $\Delta\Delta C_T$ method was used to analyze the relative changes in mRNA expression and normalized by beta-actin as previously described (35).

Bone assessments

Micro-Computed Tomography (microCT) was performed using microCT (Skyscan 1172) at 12 micron resolution using methods previously published (36). Briefly, a 1 mm region of interest starting roughly 0.5 mm from the distal end of the growth plate was used for analysis. Trabecular bone volume (BV/TV, %) and cortical porosity were quantified. Whole femur were scanned (Skyscan 1176) at 18 micron resolution to assess geometric properties at the mid-diaphysis. All CT analyses were done in accordance with standard guidelines (37).

For dynamic bone histomorphometry, undemineralized proximal tibia were fixed in neutral buffered formalin then subjected to serial dehydration and embedded in methyl methacrylate (Sigma Aldrich, St. Louis, MO). Serial frontal sections were cut 4 μ m-thick and left unstained for analysis of fluorochrome calcein labels. Histomorphometric analyses were performed using BIOQUANT (BIOQUANT Image Analysis, Nashville, TN). A standard region of interest of trabecular bone excluding primary spongiosa and endocortical surfaces was utilized. Total bone surface (BS), single-labeled surface (sLS), double-labeled surface (dLS), and interlabel distances were measured at 20x magnification. Mineralized surface to bone surface (MS/BS; $[dLS+sLS/2]/BS*100$), mineral apposition rate (MAR; average interlabel distance/10 days) and bone formation rate (BFR/BS; $[MS/BS*MAR]*3.65$) were calculated. All nomenclature for histomorphometry follows standard usage(38).

Femoral diaphysis mechanical properties were assessed in 4-point bending on a mechanical testing system (Test Resources). Bones were thawed to room temperature, hydrated

in saline, and then placed posterior surface down on bottom supports (span = 18 mm). The upper supports (span = 6 mm) were brought down in contact with the specimen's anterior surface, and then testing was conducted at a displacement rate of 2 mm/min. Force versus displacement data were collected at 10 Hz and structural parameters were determined from curves using a customized MATLAB program. Material properties were estimated using standard beam-bending equations (39).

Statistics:

Statistical analyses were conducted by first excluding outliers using ROUT ($Q = 1\%$), then checking for normality ($p < 0.05$ with Anderson-Darling Test). If normality testing was fulfilled, we used a One-Way ANOVA and, if overall ANOVA had a $p < 0.05$, we conducted within group comparisons using Dunnett's post hoc analyses, comparing CKD versus each of the other groups (NL, ALT 3mg/kg, ALT 15 mg/kg, and Calcium). If normality was not fulfilled (PTH, FGF23, cortical porosity) then the data for each group was first log transformed, normality confirmed, and then the identical analyses as above performed. The results are expressed as means \pm SD, with $p < 0.05$ considered significant (GraphPad Prism Software, La Jolla, CA).

Results:

The effect of ALT-711 on AGE-modified type I collagen matrix in vitro:

Type I collagen was incubated with or without 100 mM ribose in the presence or absence of ALT-711 for 3 weeks and the resulting collagen was qualitatively examined by confocal microscope. As shown in Figure 1, ribose led to higher amounts of collagen fluorescence suggestive of larger AGE formation (Figure 1B) compared to without ribose (Figure 1A).

However, the addition of ALT-711 at the beginning of glycation (addition of ribose) (Figure 1C) or after three weeks of ribose (Figure 1D) resulted in notably lower fluorescence.

The effect of ALT-711 on CKD-MBD biochemical parameters:

The present study utilized a slowly progressive model of CKD, the Cy/+ rat, in order to begin the treatment during mild CKD (approximately stage 3 CKD, glomerular filtration rate approximately 50% of normal) with follow up to end stage renal disease. Elevated AGE accumulation measured by skin autofluorescence is observed as early as stage 3 CKD in humans, and confers risk of kidney disease progression and cardiovascular disease (40). There was no difference in body weight between the animal groups but, as expected, the cystic kidney weight was approximately twice normal in CKD without effect of treatment (Supplemental Table 1). The biochemical analyses reflected the presence of CKD-MBD (Table 1): There was lower kidney function at the endpoint as indicated by increased plasma BUN and creatinine levels in CKD compared to Normal rats with no effect of treatment. Calcium levels were higher only in calcium drinking water treated CKD animals (to simulate calcium containing phosphate binders) relative to CKD. CKD animals had higher serum phosphorus levels compared to normal animals and treatment with calcium in the drinking water normalized these serum phosphorus levels to those of non-CKD animals. The serum levels of PTH (Table 1 & supplemental Figure 1A) and FGF23 (Table 1 & supplemental Figure 1B) were significantly higher than normal in all CKD groups. As we have previously shown (29), calcium treatment resulted in lower PTH in CKD animals. The administration of ALT-711 did not alter biochemical levels or PTH levels, but low dose led to significantly lower FGF23 levels compared to other CKD groups (Table 1 & supplemental Figure 1B).

To assess the changes in oxidative stress with ALT-711 treatment, we measured multiple markers (Table 2). There was increased oxidative stress as measured by 8-OHdG, a marker of DNA oxidation, in CKD rats compared to normal animals. Calcium, but not ALT-711 at either dose, resulted in lower 8-OHdG. There was no difference in the serum lipid peroxidation marker TBARS or inflammation marker CRP between normal and any of the CKD animal groups. Total iron and TIBC were lower in all CKD animal groups compared to normal animals with no effect of any dose of ALT or calcium on either variable. There was no difference among groups in serum total AGE levels, although it should be noted that this kit measures predominately N-(Carboxymethyl)lysine (CML).

Effect of ALT-711 on the cardiovascular system:

We have previously demonstrated that calcification occurs exclusively in the medial section of the artery(41) and therefore in this study focused on quantifying of the calcification. There was higher vascular calcification in untreated CKD compared to normal ($p = 0.01$; Figure 2A). Treatment with ALT-711 at the lower dose (3mg/kg) led to significantly lower aortic arch calcification (Figure 2A, $p < 0.002$). Interestingly, ALT-711 at the higher dose (15mg/kg) had no effect on calcification (Figure 2A). There was no difference for aorta AGE accumulation among all groups as assessed by total tissue fluorescence (Figure 2B). The expression of the receptor for AGEs (RAGE) was higher in the untreated CKD animals compared to normal controls, and treatment with either lower dose of ALT-711 or calcium reduced expression to levels of that in normal animals. The higher dose of ALT-711 had no effect on RAGE expression (Figure 2C). No differences were observed in AGR1 expression in the aorta (data not shown), a more constitutively expressed receptor. In addition, expression of NOX-2 trended higher in untreated CKD animals compared to normal animals and was reduced by low dose ALT and calcium

treatments(Figure 2D). No differences were observed in NOX-4 (data not shown) which is constitutively expressed.

In the heart, there was higher LVMI in untreated CKD animals compared to normal and only 3 mg/kg ALT-711 reduced LVMI in CKD animals (Table 3). There was no evidence of heart calcification in CKD rats (Table 3). However, there was increased RAGE expression but not NOX2 expression in heart in the CKD rats, unaffected by treatment (Table 3).

Effect of ALT-711 on bone:

There were higher levels of AGEs assessed by total fluorescence in femoral bone tissue from untreated CKD rats compared to normal animals (Figure 3); treatment with 3 mg/kg ALT-711 and calcium normalized levels while the higher dose of ALT-711 had no effect (Figure 3). HPLC (supplemental Figure 2) demonstrated decreased pyridinoline/collagen in CKD compared to normal rats, which increased with both 3 mg/kg and calcium treatment. However, there was no change in the AGE pentosidine. MicroCT assessment of proximal tibial bone demonstrated that there was lower trabecular bone volume fraction in CKD rats compared to normal animals (Figure 4A). As we have previously observed (29, 36) calcium treated CKD rats had higher trabecular bone volume fraction; however there was no effect of either ALT-711 dose on trabecular BV/TV (Figure 4A). CKD rats also had higher cortical porosity compared to normal rats and CKD rats treated with calcium (Figure 4B). Dynamic histomorphometry demonstrated increased trabecular bone formation rate in CKD rats compared to normal animals (Figure 4C). As shown in previous studies, calcium treatment reduced trabecular remodeling (29). Neither dose of ALT-711 had any effect on cortical porosity or bone formation rates. Bone mechanical analysis demonstrated that while several properties were lower in untreated CKD animals, calcium, but not either dose of ALT-711 treatment, normalized these mechanical properties in CKD rats (Table 4, supplemental Table 2 & 3).

Discussion:

In the present study we tested the hypothesis that a reduction in AGE formation of collagen, using ALT-711, would have favorable effects on the manifestations of CKD-MBD. In the CKD animals 3 mg/kg reduced arterial calcification, aorta RAGE expression, and NOX2 expression. Calcium treatment only reduced RAGE and NOX2 expression. There was no effect of ALT-711 on calcification of the heart, but there was a reduction in left ventricular mass index with the 3 mg/kg dose, presumably due to reduction in arterial calcification thereby reducing afterload and/or from the decreased in serum FGF23, a known inducer of LVH(4). In bone, there was a decrease in cortical porosity and bone formation rate with calcium as we have previously reported(29), however, ALT-711 had no effect. There was an increase in total fluorescence (a marker of total AGEs) but not the AGE pentosidine in CKD, with a decrease in bone AGE in response to normal dose ALT-711. These changes did not result in improved bone mechanical properties. The administration of either dose of ALT-711 did not affect markers of inflammation or oxidative stress which would have been expected as a result of decreased AGE levels. Taken together, the efficacy of ALT-711 appeared to predominately affect vasculature, consistent with human studies demonstrating reduced pulse wave velocity(27, 28).

We have previously shown that vascular calcification is accelerated in vitro by glucose, high phosphorus, and uremic serum (42, 43), via the de-differentiation of VSMC to an osteochondrocytic-like cell with upregulation of the osteoblast transcription factor RUNX2 is a key step. Tanikawa (44) found similar phenotypic changes and increased calcification of human VSMC cultured with glycated albumin vs. non-glycated albumin, and in serum from diabetic patients versus normal controls; the calcification was inhibited through anti-RAGE antibodies. In diabetic rats with arterial calcification induced by vitamin D and warfarin (to inactivate the

calcification inhibitor matrix gla protein), 10 mg/kg ALT-711 given in the food, reduced femoral, but not aortic, calcification and total AGE content(12). In vitro studies demonstrated that AGEs needed to bind to RAGE in order to inhibit calcification. Activation of RAGE induces multiple cell signaling pathways that have been shown to be involved in arterial calcification in vitro including JAK/Stat, NFK β , MAPK and ERK signaling(6). In addition, activation of RAGE increases oxidative stress, mediated in part by NOX(45). In the current study we found that 3 mg/kg of ALT-711 decreased expression of RAGE and decreased NOX expression in the aorta.

Activation of RAGE signaling is known to increase oxidative stress and systemic oxidative stress activates cellular RAGE expression(11, 46). We observed increased oxidative stress in our CKD animals compared to normal littermates, but no effect of ALT-711 to reduce oxidative stress. In the adenine-induced CKD and 5/6th nephrectomy and obese rat models, arterial calcification was prevented by the anti-oxidants tempol(47) and Vitamin E(48) similar to our findings of decreased calcification at 3 mg/kg of ALT-711. In contrast, we failed to see any effect on vascular calcification with N-acetylcystine in the Cy/+ model (unpublished data) or high dose ALT-711. The discrepant data could be due to differences in the acute versus chronic nature of CKD and/or the high phosphate diets used in the adenine and 5/6th nephrectomy animal models, or that high dose ALT-711 and N-acetylcystine are ineffective in reducing oxidative stress or RAGE expression in CKD. Thus, in the present study, the data suggests that the effects of 3 mg/kg ALT-711 are mediated by RAGE and subsequent intracellular ROS rather than decreased systemic oxidative stress.

Similar to the efficacy of 3 mg/kg ALT-711 in reducing arterial calcification, we did see an increased total fluorescent AGE content in bone from CKD rats that was reduced by 3 mg/kg

ALT-711. We have previously observed increased pentosidine, one specific AGE found in bone, at an early stage of disease (30 weeks), in our CKD (Cy/+) animal model together with impaired bone mechanics (49), and Mitome also found increased pentosidine in bone from dialysis patients compared to controls and impaired mineralization(14). In the current study, we found increased total bone fluorescence (AGEs), but no change in pentosidine in bone at 35 weeks, with no improvement in bone mechanics with either dose of ALT-711. Although we can't explain the discrepancy between this outcome and our past work, it is worth noting that pentosidine represents just one of several AGEs in the bone and thus changes in AGEs could be driven through differential processes that are not completely understood. In addition, Thomas et al recently demonstrated that the bone content of non-fluorescent AGEs, such as CML, may be 40-100 times more common than pentosidine (50). These data highlight the complexity of AGE accumulation in bone and unfortunately leave more questions than answers with respect to AGE changes in CKD.

The present study showed increased cortical porosity and low trabecular bone volume in the CKD animals; these were both reversed with calcium-induced lowering of PTH as in our previous studies but not ALT-711. Although there was no change in PTH with ALT-711, the FGF23 levels were reduced by 45 to 52% with 3 mg/kg ALT-711 doses. This may have been secondary to a direct effect of RAGE on osteoblasts/osteocytes to reduce FGF23(51), or indirect effects of local inflammation to reduce FGF23 production from osteoblasts(52). The latter seems most likely given no effect on bone remodeling. Another possibility is that changes in the AGE content of collagen in response to ALT-711 were enough to affect osteocyte cell function but not significant enough to affect biomechanics. More studies would be needed to understand the marked reduction in FGF23 levels in CKD animals treated with ALT-711.

Interestingly, the higher dose of ALT-711 was ineffective compared to the 3 mg/kg dose for all measures for reasons that are not clear. ALT-711 is thought to be an AGE breaker, chemically cleaving the carbon-carbon bond in α -dicarbonyl containing cross-linked structures and our in vitro studies (Figure 1) suggest that ALT-711 can function as both an inhibitor of AGE formation and a breaker of already formed AGEs. However, this mechanism of breaking is controversial(53) and some authors believe the primary effect of ALT-711 is by chelating activities of metal-catalyzed oxidation(54). In order to further understand the lack of a dose response, we performed a series of studies. First, it is worth noting that in the aorta, only the 3 mg/kg dose reduced calcification and RAGE/NOX expression, raising the possibility that RAGE is differentially expressed at the two doses and this signaling difference explains the lack of dose efficacy. Second, we confirmed there was no solubility issue with the 3 versus 15 mg/kg dose. Third, there was no increased mortality, or local injection site irritation to suggest altered dose delivery. Fourth, we measured iron levels to test the hypothesis that there may be differences in the metal-catalyzed oxidation and did not find any differences. Unfortunately, we did not collect the samples in a way to measure copper levels. Finally, we conducted dynamic bone histomorphometry to determine if the doses differentially affected trabecular bone remodeling, yet there was no significant effect. Thus, at this time we cannot explain the lack of dose effect at 15 mg/kg, but hypothesize that there may be differences in cell signaling induced by the two doses studied. Further work is needed to fully understand the lack of dose-dependent effects.

In summary, 3 mg/kg ALT-711 reduced aorta expression of RAGE, NOX2, and arterial calcification, decreased serum FGF23 levels, and reduced total bone AGE. The high dose (15 mg/kg) of ALT-711 had no effect on measured parameters. There was no improvement in the overall mechanical bone quality at either dose of ALT-711 as assessed by mechanical testing.

These results highlight the need to better measure specific types of AGE proteins at the tissue and cell signaling in order to fully elucidate the impact of AGEs on CKD-MBD.

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Figure legends:**Figure 1: *The effect of ALT-711 on AGE formation on collagen matrix in vitro.***

Type I collagen was incubated with or without ribose (100 mM) in the presence or absence of ALT-711 (2 mM) for 3 weeks, or ALT-711 was added after ribose-collagen incubation for 3 weeks and then incubated for additional 7 days. AGE-collagen was examined qualitatively by fluorescence (356 nm excitation /440 nm emission) by confocal microscopy. The results demonstrated an apparent increase in AGE formation with ribose (B) compared to control (A). Addition of ALT-711 at the beginning of glycation (C) or after three weeks of ribose (D) prevented and reduced, respectively, AGE formation.

Figure 2: *The effect of ALT-711 on aortic expression of RAGE and NOX and vascular calcification in CKD rats:*

There was increased vascular calcification in CKD animals (A) and treatment with ALT-711 at normal dose (3mg/kg) but not the higher dose (15mg/kg) significantly decreased calcification in CKD rats (A). There was no difference in total AGE accumulation in all animal groups (B). Expression of the RAGE in aorta was increased in CKD animal compared to normal rats (C) and treatment with 3 mg/kg ALT-711 or calcium significantly reduced the expression of RAGE in CKD rats (C). In addition, aortic NOX2 expression was increased in CKD animals compared to normal rats (D) and treatment with 3 mg/kg ALT-711 or calcium significantly reduced the expression of NOX2 in CKD rats (D). Data are shown as mean \pm SD (n=10-13 rats each group). One-Way ANOVA, and if $p < 0.05$, Dunnett's multiple comparison test for group vs CKD with p value shown in graph.

Figure 3: *The effect of ALT-711 on AGE accumulation in bone in CKD rats:*

The demineralized femur bone tissues were then hydrolyzed in 6 M HCL and total AGE content was determined using fluorescence microplate reader and normalized by the collagen content for the sample. There was increased accumulation of AGE in femoral shaft from CKD rats compared to normal rats. Treatment with ALT-711 at normal dose (3mg/kg) or calcium reduced bone AGE accumulation in CKD rats. However, ALT-711 at high dose (15mg/kg) had no effect. Data are shown as mean \pm SD (n =10-13 rats each group). One-Way ANOVA, and if $p < 0.05$, Dunnett's multiple comparison test for group vs CKD with p value shown in graph.

Figure 4: *The effect of ALT-711 on bone architecture in CKD rats:*

The bone architecture was analyzed by MicroCT. The results demonstrated that (A) trabecular bone volume (BV/TV) was lower in CKD rats compared to either Normal rats or CKD rats treated with calcium. Cortical porosity (B) and Bone Formation rate (BFR/BS; C) were higher in CKD rats compared to Normal rats or CKD rats treated with calcium. Data are shown as mean \pm SD (n =10-13 rats each group). One-Way ANOVA, and if $p < 0.05$, Dunnett's multiple comparison test for group vs CKD with p value shown in graph.

Tables

Table 1: Serum Biochemistries:

Treatment groups	BUN (mg/dL)	Creatinine (mg/dL)	Calcium (mg/dL)	Phosphorus (mg/dL)	PTH (pg/ml)	FGF23 (pg/ml)
Normal	18.20±3.84*	0.74±0.22*	8.02±1.07	3.47±0.40*	145±44*	412±191*
CKD	48.80±8.22	1.51±0.28	8.51±1.34	8.33±2.71	1237±579	5751±1382
CKD/ALT-711 (3 mg/kg)	49.50±4.66	1.46±0.48	7.52±1.68	8.02±2.41	1285±738	2499±516#
CKD/ALT-711 (15 mg/kg)	49.45±6.86	1.32±0.20	8.73±1.52	6.93±1.75	1137±765	3189±598
CKD/3% Ca	46.20±7.59	1.18±0.22*	9.92±1.73#	5.46±1.40*	57±32*	11008±2955

PTH: parathyroid hormone; FGF23: fibroblast growth factor 23. Data are shown as mean ± SD (n =10-13 rats each group). All of the measures were significantly different by ANOVA. Within group comparisons vs. CKD by Dunnett's test: *p < 0.001, vs. CKD, # p < 0.05 vs. CKD.

Table 2: Serum oxidative stress/inflammation markers

Treatment groups	AGE(ng/ml)	8-OHdG (ng/ml)	TBARS (μg/ml)	CRP (μg/ml)	Total iron (μmol/L)	TIBC (μg/dL)
Normal	70.71±6.22	13.28±1.97*	39.09±3.97	595.1±37.73	55.8±10.06#	645.5±21.12*
CKD	77.80±9.60	21.8±4.29	42.26±2.29	589.8±57.07	43.46±4.91	530.3±40.72
CKD/ALT-711 (3 mg/kg)	73.15±8.74	20.12±6.40	34.18±3.16	582.3±59.08	41.18±3.10	543.5±14.34
CKD/ALT-711 (15 mg/kg)	82.52±9.32	23.98±4.01	37.04±0.99	590.9±89.99	39.00±12.28	544.5±25.7
CKD/3% Ca	76.82±8.92	15.29±4.23#	39.6±5.52	588.9±80.65	48.98±7.46	546.5±27.96

AGE: Advanced glycation end products; 8-OHdG: 8-hydroxyguanosine; TBARS: Thiobarbituric Acid Reactive Substances; CRP: C-reactive protein; TIBC: total iron binding capacity. Data are shown as mean ± SD (n =10-13 rats each group). 8-OHdG, Iron, and TIBC were significantly different by ANOVA. Within group comparisons vs. CKD by Dunnett's test: *p < 0.01 vs. CKD, # p < 0.05 vs. CKD.

Table 3: Cardiovascular parameters

Treatment groups	LVMI	Heart calcification (μmol/g)	Heart RAGE/β-actin	Heart NOX2/β-actin
Normal	2.82±0.16*	0.79±0.10	0.89±0.26#	1.04±0.18
CKD	3.45±0.38	0.76±0.17	1.46±0.58	0.98±0.23
CKD/ALT-711 (3 mg/kg)	3.10±0.33#	0.78±0.21	1.38±0.43	1.09±0.28
CKD/ALT-711 (15 mg/kg)	3.21±0.27	0.80±0.20	1.37±0.37	1.13±0.66
CKD/Ca	3.33±0.30	0.81±0.17	1.31±0.41	0.98±0.24

LVMI: Left ventricular mass index. Data are shown as mean ± SD (n =10-13 rats each group).

Only LVMI was significantly different by ANOVA. Within group comparisons vs. CKD by Dunnett's test: *p < 0.01 vs. CKD #p <

0.01 vs. CKD. 5, NL vs. CKD; #p<0.05, CKD vs. CKD with treatment.

Table 4: Cortical bone geometry and mechanical properties

Treatment groups	Cortical area (mm ²)	Cortical thickness (μm)	CSMIp (mm ⁴)	Ultimate Force (N)	Total Displacement (mm)	Stiffness (N/mm)	Total Work (mJ)	Ultimate Stress (MPa)	Modulus (GPa)	Toughness (MPa)
Normal	6.67 ± 0.29	536 ± 12	18.99 ± 1.52	230 ± 15*	699 ± 58	495 ± 44*	97 ± 13	153 ± 7*	4.78 ± 0.41	4.46 ± 0.59
CKD	6.30 ± 0.41	489 ± 69	18.34 ± 1.31	167 ± 34	704 ± 163	428 ± 45	77 ± 32	127 ± 21	4.86 ± 0.48	3.90 ± 1.50
CKD/ALT-711 (3 mg/kg)	6.25 ± 0.39	503 ± 32	17.92 ± 2.43	180 ± 20	713 ± 98	443 ± 36	82 ± 21	134 ± 19	4.90 ± 0.63	4.12 ± 0.87
CKD/ALT-711 (15 mg/kg)	6.20 ± 0.47	489 ± 60	17.74 ± 1.41	166 ± 27	755 ± 184	434 ± 36	82 ± 25	124 ± 18	4.80 ± 0.55	4.18 ± 1.27
CKD/Ca	6.76 ± 0.23	541 ± 17	18.81 ± 1.84	225 ± 15*	654 ± 57	499 ± 44*	86 ± 12	163 ± 11*	5.52 ± 0.63*	4.09 ± 0.59

CSMIp = polar cross sectional moment of inertia. Data are shown as mean ± SD (n =10-13 rats each group). Ultimate force, stiffness, ultimate stress, and modulus were significantly different by ANOVA. Within group comparisons vs. CKD by Dunnett's test: *p < 0.01 vs. CKD, #p < 0.05 vs. CKD.

Figure 1: ALT-711 inhibited AGE formation and break down AGE in ribose-collagen coated dishes determined by confocal microscopy

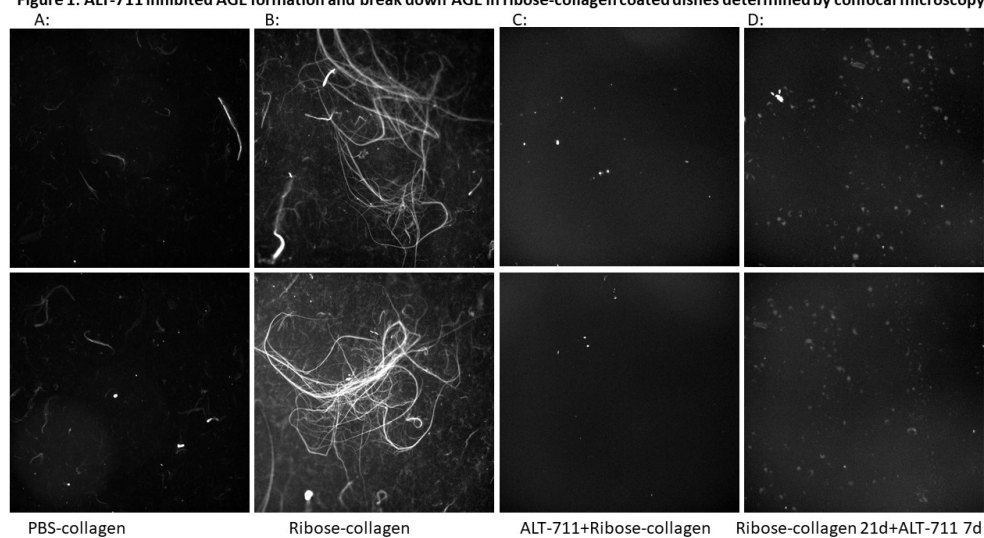


Figure 2

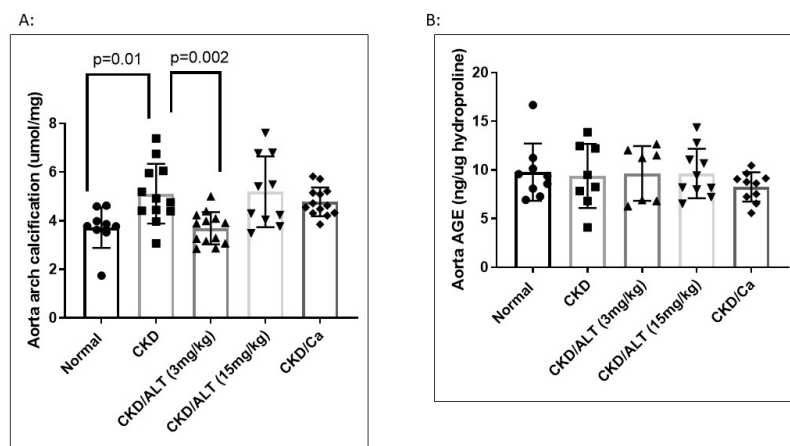


Figure 2

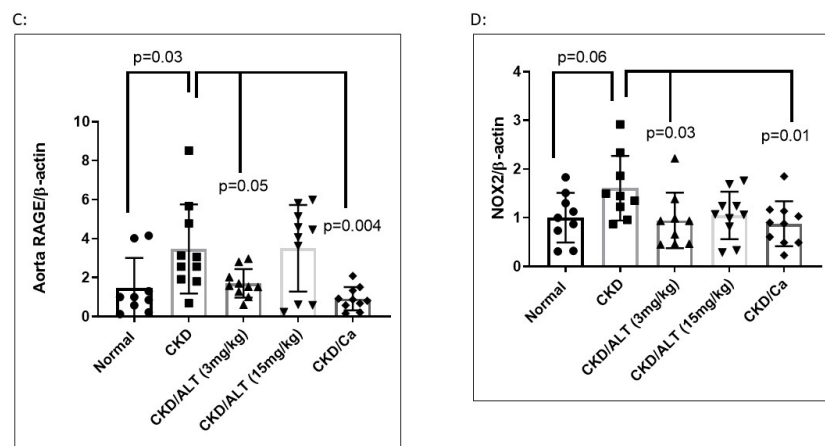


Figure 3

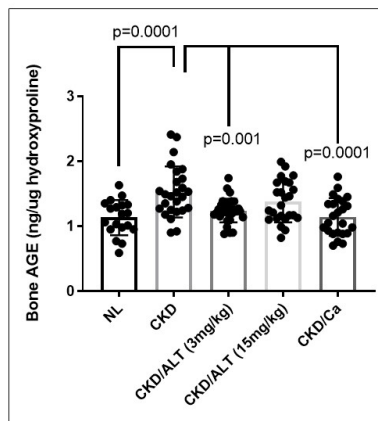


Figure 4

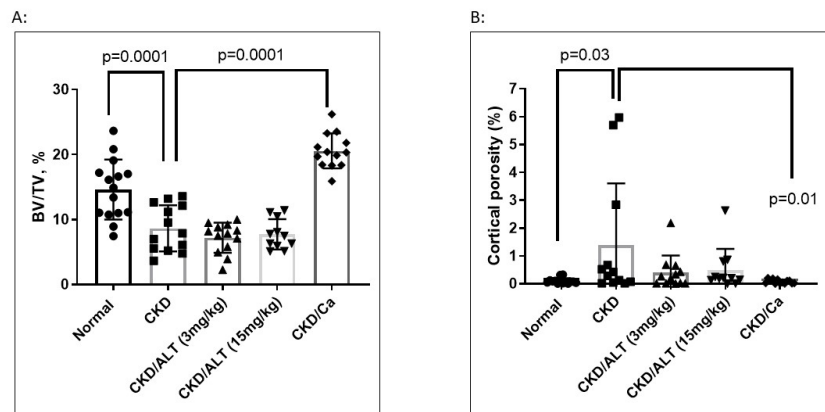


Figure 4C

